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ENTATION PAGE

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ARO 22367.11-LS	2. GOVT ACCESSION NO. / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle): The Rapid Detection of Bacteria and Other Micro-organisms: A Basic Study in the Application of Resonance Raman and Time-Resolved Fluorescence Spectroscopies.	5. TYPE OF REPORT & PERIOD COVERED: 10-15-84 to 2-14-88
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): Wilfred H. Nelson	8. CONTRACT OR GRANT NUMBER(s): DAAG29-85-K-0005
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Chemistry Department, University of Rhode Island, Kingston, RI 02881	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS: U. S. Army Research Office, Post Office Box 12211, Research Triangle Park, NC 27709	12. REPORT DATE: April 15, 1988 13. NUMBER OF PAGES: 21
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report): Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

NA

18. SUPPLEMENTARY NOTES

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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Detection, Identification, Bacteria, Spora, Resonance Raman, Spectra

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Resonance Raman Spectra have been obtained for a variety of chromobacteria using low power 488 nm excitation. Spectra are simple, of high quality, and useful for identification purposes at the species level. Raman microprobe studies show conclusively that spectra can be obtained from single cells in pure cultures or in mixed cultures without need for separation.

Extensions of the study have been made to representative colorless gram-negative and gram-positive bacteria of various genera and species using ultra-

1 open

violet excitation of 251, 242, 231 and 222 nm. The ultraviolet excited resonance Raman spectra are also of high quality, and of greatest significance allow the selective excitation of taxonomically significant cell components. While identification will probably be possible on the genus level using any of the above UV wavelengths excitation of specific markers at given UV wavelengths holds the promise of greater specificity. Excitation of 222 nm especially seems to promise selective excitation of cell wall components.

Spora other than bacteria have been studied as well. Pollen, mold spores, bacterial spores, algae and viruses all give spectra but only viruses and bacterial spores appear to give intense UV Resonance Raman spectra. Pollen and mold spores are nearly silent and, consequently, should not interfere with the detection of bacteria or spores. Of major importance is the observed absence of fluorescence interference at all of the above UV wavelengths.

The primary fluorescence of bacteria has been studied in detail to determine its potential in rapid detection. Excitation and emission spectra have been determined over the range 290-650 nm for a variety of organisms. We have determined fluorescence lifetimes at 340, 430, 487 and 514 nm for S. epidermidis, P. fluorescens, E. cloacae, E. coli and B. subtilis. Fluorescence contributions have been assigned in part to tryptophan, pteridines, related flavins and pyridine coenzymes. Lifetimes in the (1-10) nanosecond range are characteristic for the various organisms, but except for P. fluorescens vary over limited lifetime ranges suggesting limited specificity for fluorescence as a means of detection and identification.

A. The Problem Studied

Often, important decisions relating to the presence of pathogens must be made before the results of conventional microbiological tests are available. To safeguard the public, a number of very effective detection techniques have been developed, some of which are highly effective and specific. However, commonly, no single test will allow the identification of an unknown organism. Rather, complex series of tests are required which may be difficult to interpret and which often require many days or even weeks to complete.

In the event an enemy released a biologically active material over a city or a military base, present methods of detection and identification would be too slow to be of immediate help to decision-makers or potential victims. To address this problem we have attempted to answer a number of questions related to the feasibility of using resonance Raman spectroscopy and fluorescence lifetime spectroscopy as a means of rapidly detecting and identifying microorganisms.

Very specifically we have attempted to determine the sensitivities of these methods, i.e., detection limits, and have tried to determine to what extent microorganisms can be rapidly and unambiguously identified by these spectroscopic means.

B. Summary of the Most Important Results

1. Fluorescence Studies

The most important results of our fluorescence studies have already been reported in detail(1-3). We have determined that fluorescence excitation and emission spectra are within broad limits characteristic of various types of bacteria(1). Primary fluorescence of bacteria can be intense and specific. However, the spectra of most organisms have many similarities and interference and interpretation problems are great.

Fluorescence lifetime studies hold more promise, we have noted that protein tryptophan fluorescence lifetimes are characteristic for most bacteria(2) and that sensitivity is high because of the high quantum efficiency of the tryptophan fluorescence. However, differences in tryptophan lifetimes between types of organisms are small.

Of somewhat grater promise in terms of specificity is the fluorescence observed between 350-500 nm. The observation that bacteria show characteristic sets of lifetimes suggests that the measurements of fluorescence lifetimes may be helpful in the rapid characterization of bacteria. Lack of specificity with the fluorescence technique is a serious problem, however. It appears that if fluorescence is to be used in rapid detection it will be as a pre-screening method or as a rapid, non-specific means of warning of the potential presence of protein containing substances.

2. Raman Studies

Central to this study has been the objective of determining just how valuable Raman spectroscopy can be in the rapid detection of microorganisms. Very early we began to enjoy major successes in applying this method of detection

and identification to bacteria. Using chromobacteria excited by conventional Ar^+ laser lines we have been able to obtain resonance Raman microprobe spectra of individual cells from pure cultures(4)[Figure 1] and from mixed cultures(5). This last finding was especially exciting since it demonstrates the very high sensitivity and selectivity possible with resonance Raman detection. The same article reported substantial differences in the resonance Raman spectra of chromobacteria at the species level.

Of course, it is well known that most bacterial components of chemo taxonomic importance are colorless(6) or very faintly colored. Consequently, they can be resonance Raman excited only in the ultraviolet. Because of the presence of aromatic amino acid fluorescence, studies are limited to the wavelengths below 260 nm(7). However, below 255 nm fluorescence background becomes very low and essentially negligible compared to the resonance Raman scattering.

Because lasers needed to produce the excitation below 260 nm have been state-of-the-art devices progress in determining these spectra has been more difficult. Still progress has been relatively rapid. Two years ago we were able to report at an ARO conference at Cashiers, N.C. the first deep-UV-excited Raman spectra of bacteria(7). Spectra are intense, characteristic of protein and nucleic acid content, and of more importance from the standpoint of detection, characteristic for different organisms. We quickly extended our study to 251 nm, 231 nm and 222 nm(8). To our delight we found that fingerprinting seems possible at all the wavelengths studied. At 251 nm the spectra seem to be due to nucleic acids primarily with quinones and amino acids of lesser import. At 232 nm the spectra reflect primarily aromatic amino acids while at 222 nm a wider range of components can be observed.

Of course, simply fingerprinting bacteria spectroscopically is not our objective. If spectra are to be used for identification the relation between spectra and the bacterial composition must be detailed. Because of the complexity of bacteria this is not a simple problem. Still the fact that bacteria are characterized by chemotaxonomic markers is well documented(6). We have been encouraged to note that it is possible to pick wavelengths which allow very specific components to be excited selectively(8).

For example, it is possible to excite dipicolinates in Bacillus spores almost to the exclusion of tryptophan components even though conventional spectra are similar. Huge differences in the spectra of gram-negative and gram-positive organisms appear associated with gross cell wall structural differences. At 222 nm especially vast differences in spectra appear which we believe can be correlated with taxonomically significant differences in cell wall compositions. We are trying to determine just which spectral features are associated with taxonomy alone. Toward that end we are recording the spectra of taxonomic markers such as quinones. Representative data for bacteria are presented below.

Figure 2 shows spectra obtained at 250.96 nm excitation. Cells in pure culture were studied by flowing them rapidly in 0.01 M phosphate buffer at pH 6.8 through the laser beam in a 3 mm i.d., 4 mm o.d. quartz tube. The spectra represent the sum of 3 to 5 scans. Typically, scans were made over a period of 10-20 minutes, and the data averaged so long as spectra did not show changes from scan to scan. Presently, data can be obtained in a matter of seconds. Our instrumentation, including flow sampling methods and cultural methods have been described elsewhere(7). Strong peaks associated with adenine, quanine, thymidine and uridine are present. There is little evidence of cytosine contributions at this wavelength.

Very substantial differences in the intensities of the nucleic acid peaks are noticed for the different organisms. The relative peak heights probably in part reflect differences in the average value of base pair ratios for the various organisms. Quantitative assessment of peak height ratios on the basis of base pair ratios is not a simple problem, however, since both ribosomal RNA and DNA probably contribute substantially. In addition electronic effects associated with stacking or other environmental effects may change overall resonance Raman peak intensities and their related peak height ratios.

Figure 3 shows comparable resonance Raman spectra excited at 230.59 nm. The most significant aspect of these spectra is the near total dominance of protein tryptophan and tyrosine peaks. Surprisingly important differences still exist between spectra. These may be the result of substantially different tryptophan tyrosine ratios in different organisms' proteins or more likely reflect different efficiencies of excitation of tryptophan and tyrosine in various proteins in different cell locations. Apparently, because the proteins in the cell wall regions very-strongly absorb and scatter 230 nm radiation, there is relatively very little contribution from nucleic acids even though nucleic acids are known to give strong resonance Raman spectra when excited alone at 230 nm even if absorption is not strong. Absorption and scattering by cell wall proteins and enzymes very likely reduces the amount of UV light incident on nucleic acids.

Figure 4 displays resonance Raman spectra excited at 222.5 nm. These spectra like those obtained at 230.6 nm show only very slight contributions from nucleic acids. They are dominated by protein amino acid peaks. For example the extremely intense 1484 cm^{-1} peak due to guanine-adenine observed in 242 and 251 nm excited spectra is replaced by another strong band at 1469 cm^{-1} probably due in part to proline(9). What appears to be an adenine peak at 1580 cm^{-1} is barely

present in gram-negative organisms and has disappeared completely for the gram-positives. Perhaps the thicker peptiodoglycan layer of the gram-positives prevents the effective excitation of the nucleic acid in those organisms with 222 nm light.

Strong features around 1660 cm^{-1} appear to be an important part of 222 nm excited spectra. It is possible that such peaks which are most evident for S. epidermidis are associated with amide I vibrations. An increase in the amide I mode intensities is expected in resonance Raman spectra as the exciting wavelength is reduced toward 200 nm(10). The greater intensity of these peaks for the gram-positive organisms may be due directly to the large amounts of peptidoglycan present in the outer cell wall.

Very substantial differences in the 900-1400 cm^{-1} region appear to be due to lesser peaks of tyrosine, tryptophan or phenylalanine. Still other peaks appear which may be due to other components not yet identified.

Figure 5 compares spectra of Escherichia coli excited at 251.6, 242, 231 and 222 nm. At these intervals huge differences in spectra are observed due to selective resonance Raman excitation of the various bacterial components. This figure emphasizes one of the major advantages of resonance Raman spectroscopy in bimolecular studies, selectively. It is anticipated that once the resonance Raman spectra of the various taxonomic marker compounds of bacteria are well characterized, it will be possible to devise strategies to maximize spectral differences between the specific types of organisms. Because all excitation frequencies between 190-255 nm should give rise to specific spectra dependent upon cell composition and structure, it is believed that with further development UV excited resonance Raman spectroscopy should provide a valuable means for rapid detection and identification of microorganisms. Because below 260 nm there is

essentially no fluorescence background, sensitivity is high. Because spectra are extremely sensitive to the exciting wavelength used, selectivity of excitation is surprisingly great. Selectivity may also be enhanced by virtue of the cells heterogeneity, which may allow selective excitation of cell regions dependent upon the extent of laser light penetration. For all these reasons, UV resonance Raman spectroscopy appears to be remarkable sensitive and selective technique capable of characterizing even as highly complex a system as a living bacterial cell.

Quite obviously if airborne bacteria are to be identified attention must be paid to other materials likely to be present in the atmosphere. Figure 6 compares spectra of B. cereus spores with those of vegetative cells and calcium dipicolinate. It is satisfying to note the major spectral differences associated with spores as opposed to the vegetative cells. It is even more satisfying to understand that the differences at 242 nm are due primarily to dipicolinate which is being selectively excited at 242 nm. That is to say, the spores' spectra at 242 nm are distinct because of the selective excitation at that wavelength of calcium dipicolinate which is found in characteristic substantional amounts in these bacterial spores but not in vegetative cells.

Even greater spectral differences are found for mold spores, pollen and algae. Because of the composition of their cell walls, especially, UV resonance Raman intensities tend to be rather low. Spectra shown in Figure 7 indicate that there will be some potential interference with bacterial spectra in the region near 1600 cm^{-1}, but, in the main, spectra of these species tend to be very weak in all other energy regions and should not pose interference problems even from spectra of mixtures.

Conclusions

It is asserted that our studies demonstrate that UV excited resonance Raman spectra of atmospheric spora are capable of sufficient detail and sensitivity to allow easy differentiation of bacteria, pollen, mold spores, algae and bacterial spores. Furthermore, because spectra directly reflect the presence of taxonomic markers the prospects for obtaining spectra capable of identifying bacterial genus and even species are excellent. Obviously much remains to be done. Our information on the spectra of taxonomic markers is not as extensive as it needs to be. Also we are just beginning to address the problems of detection of single cells. In single cell studies especially we must consider most carefully the problems associated with a cell's history to insure that spectral differences used for identification are related to taxonomy alone.

Results of fluorescence studies while encouraging in some respects do not seem to present the obvious opportunity for rapid exploitation that the Raman studies show. While the sensitivity of fluorescence techniques in principle is even better than that of the resonance Raman method, the fluorescence method suffers from poorer specificity. Perhaps of greater concern is the difficulty of directly assigning fluorescence spectral components to specific fluorphores of taxonomic significance. In contrast the link between resonance Raman spectra and bacterial taxonomy seems to be a direct one.

Very recently there has been a technological breakthrough which should give even greater momentum to this work. Until now it has been necessary to use high power, expensive Nd-Yag pulsed lasers with dye lasers and mixing crystals or Raman shifting to generate deep UV frequencies.

Last month Spectra Physics announced that they will market a relatively simple C-W laser with output in the deep UV range. This not only points to the

development of low cost laser sources, but will make the basic research much easier to accomplish. Especially it will make possible the development of a UV microprobe capable of single cell detection. Until now this was not practical because the focused high energies of the Yag pulse destroyed samples and optics. Equally exciting is the essential low cost and miniaturization possible with argon laser technology coupled with the recent availability of beta barium borate crystal technology.

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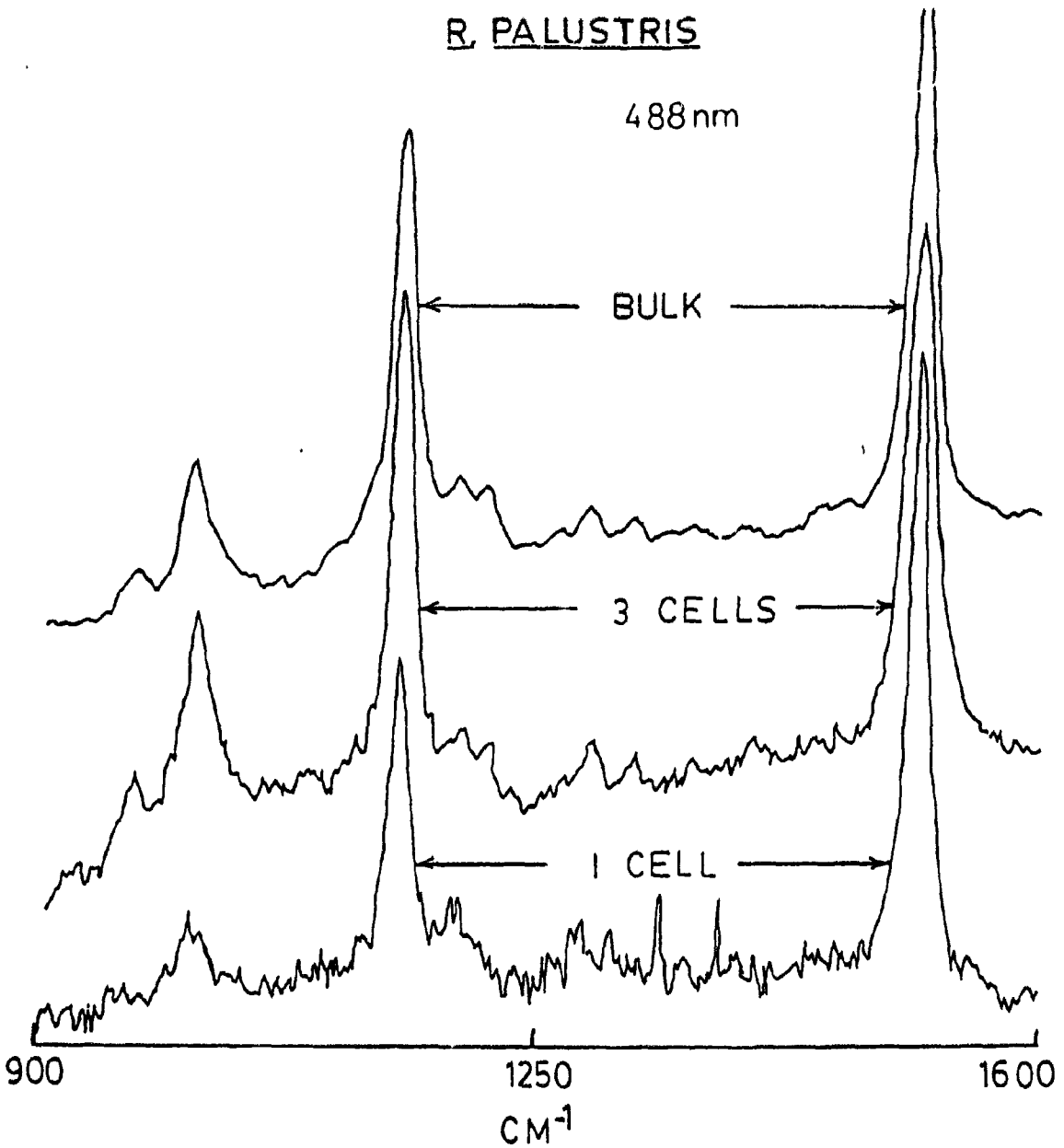

Fig. 1. Resonance Raman spectra obtained with 488 nm excitation of R. palustris (single scan).

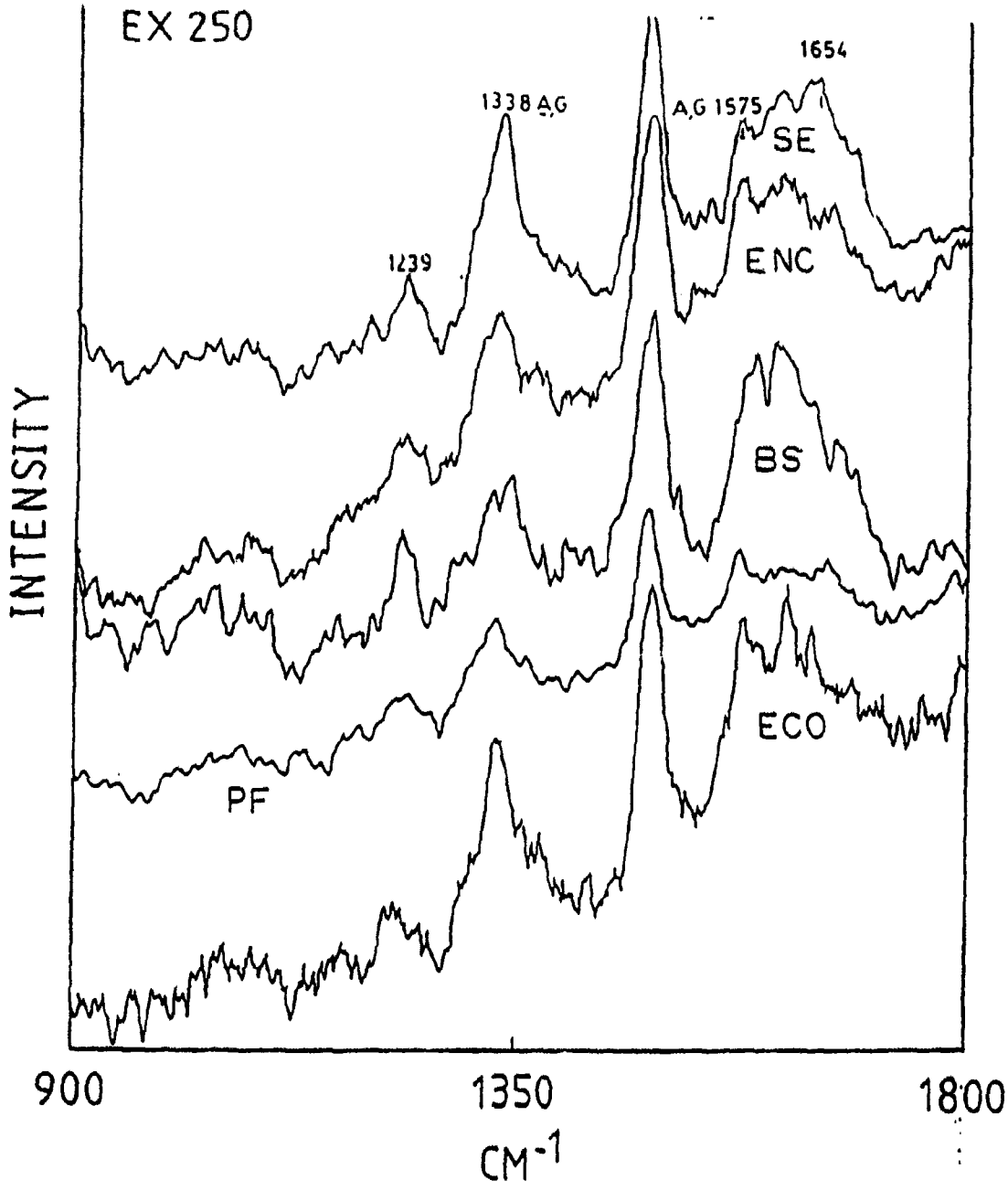

Fig. 2. Resonance Raman spectra obtained with 250.96 nm excitation obtained with 250.96 nm excitation are the sums of three scans. Bacterial spectra belong to Staphylococcus epidermidis (SE), Enterobacter cloacae (ENC), Bacillus subtilis (BS), Pseudomonas fluorescens (PF) and, Escherichia coli (ECO).

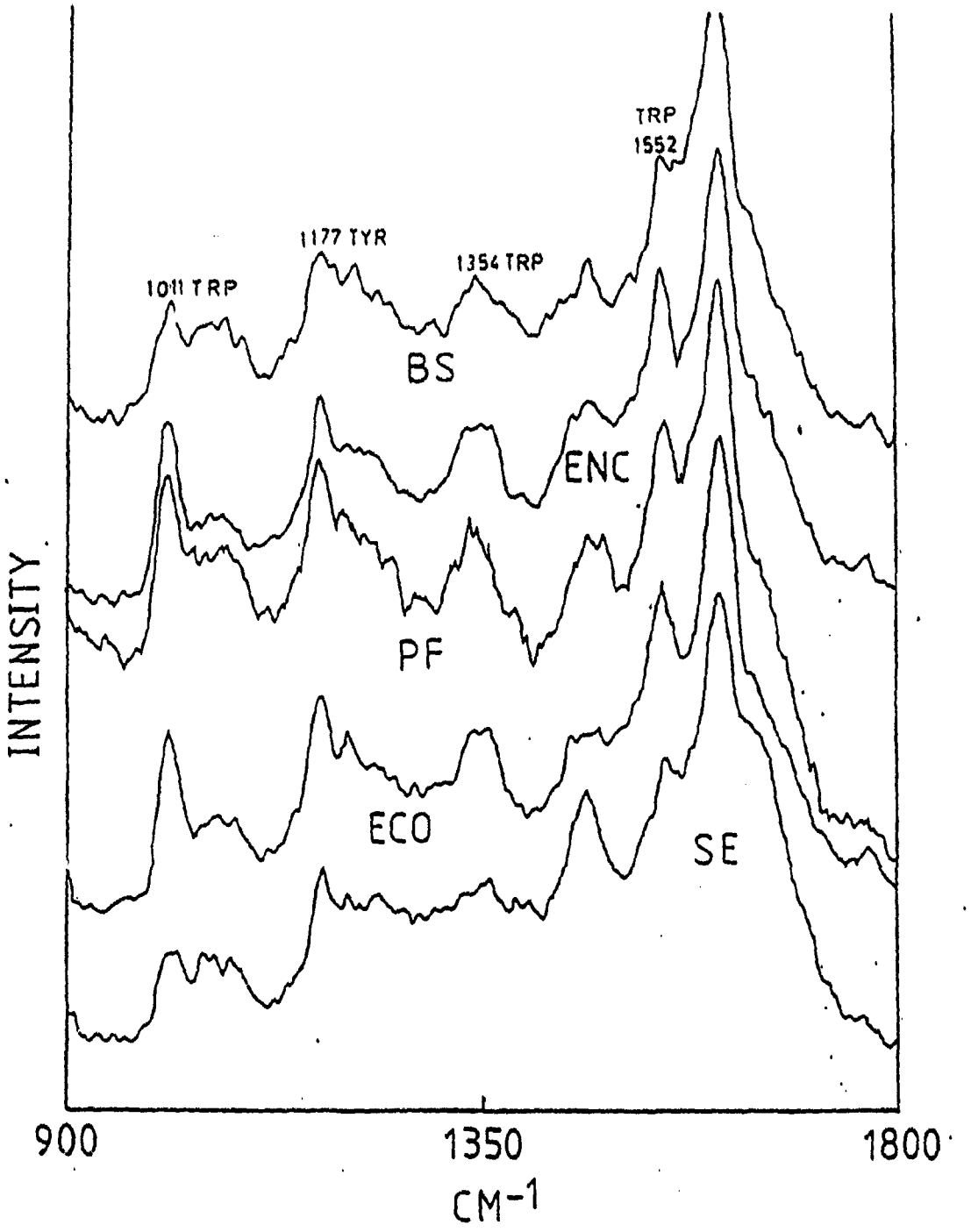

Fig. 3. Resonance Raman spectra of five bacteria obtained with 230.590 nm excitation, as sums of five scans.

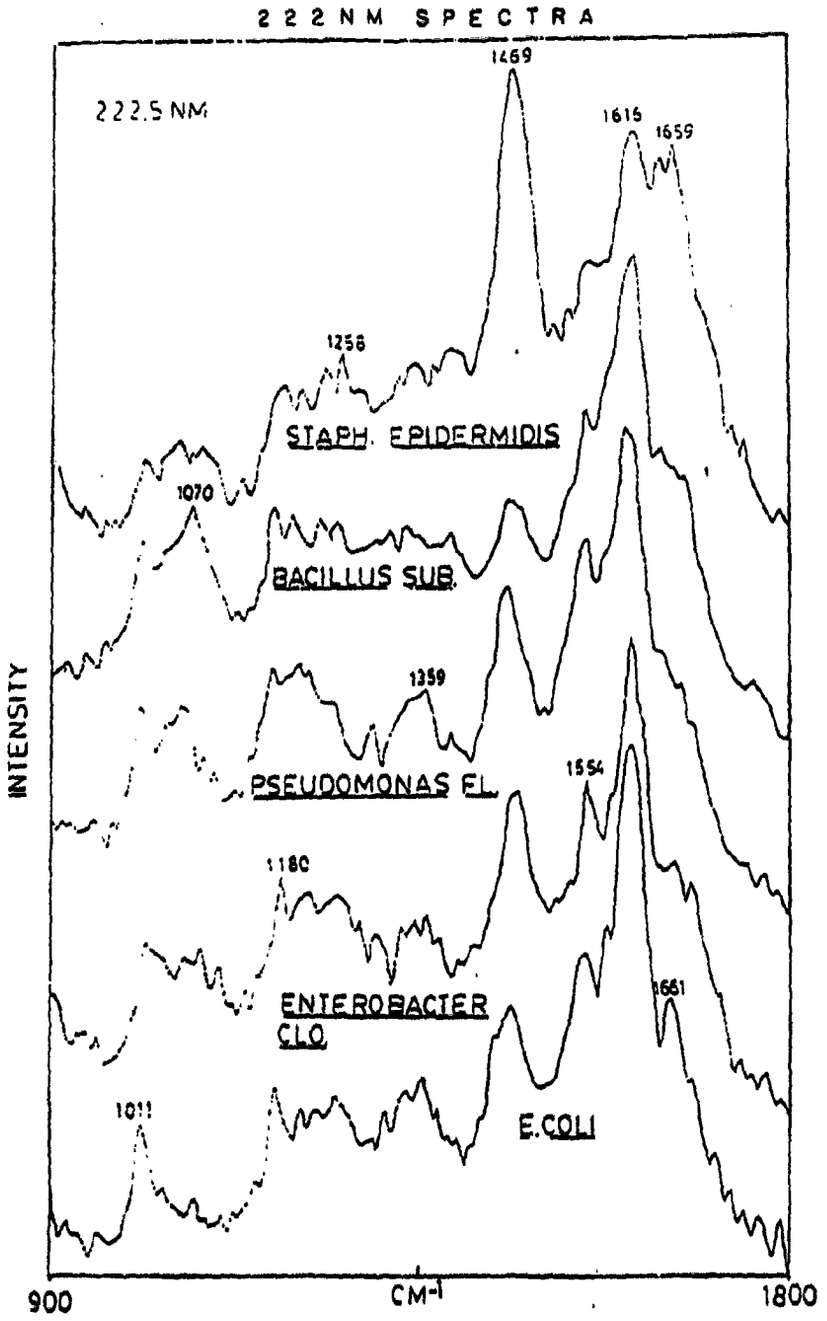

Fig. 4. Resonance Raman spectra of five bacteria obtained with 222.5 nm excitation, as sum of three scans.

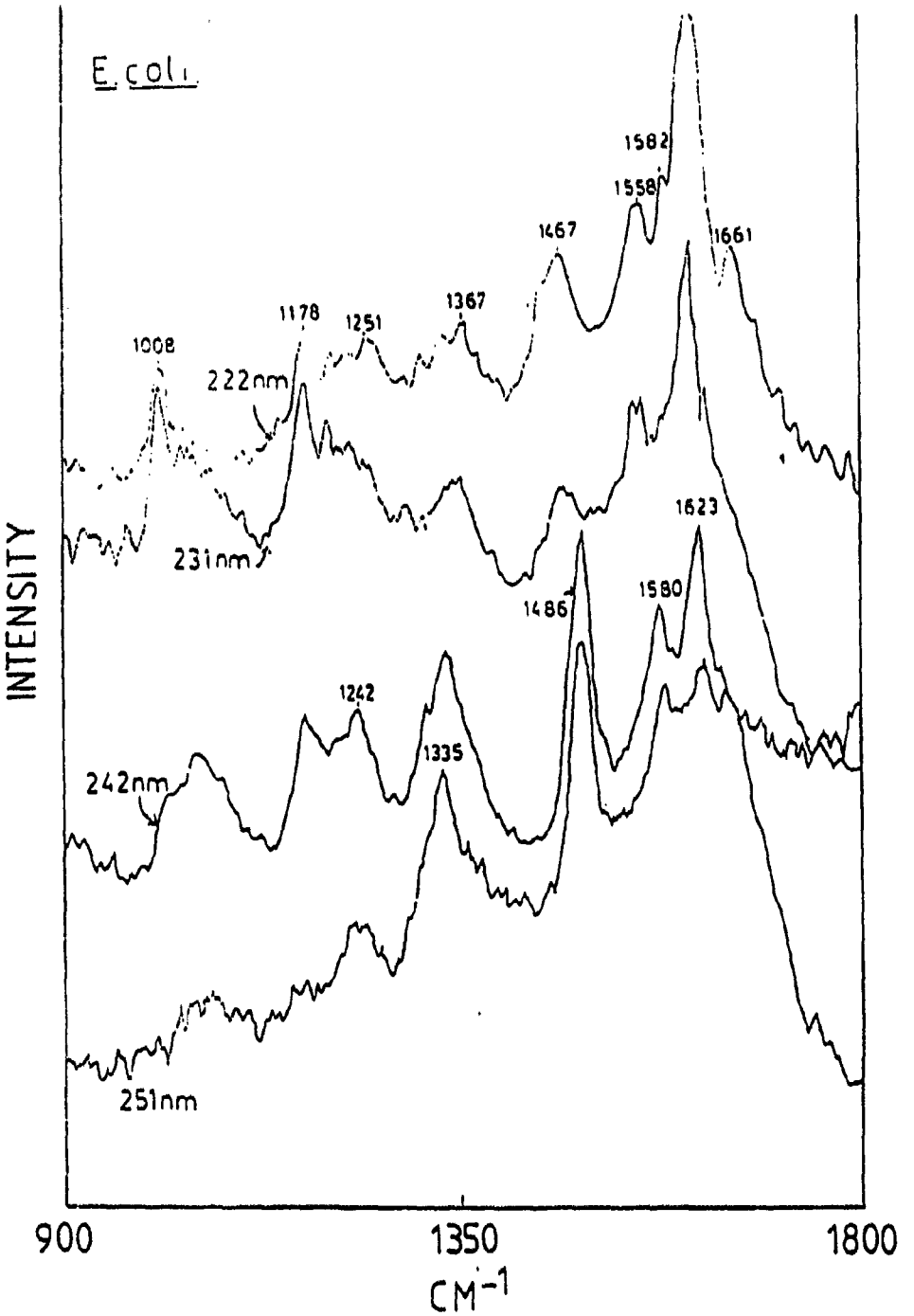

Fig. 5. A comparison of E. coli resonance Raman spectra excited at 222.50, 230.59, 242.40 and 250.96 nm.

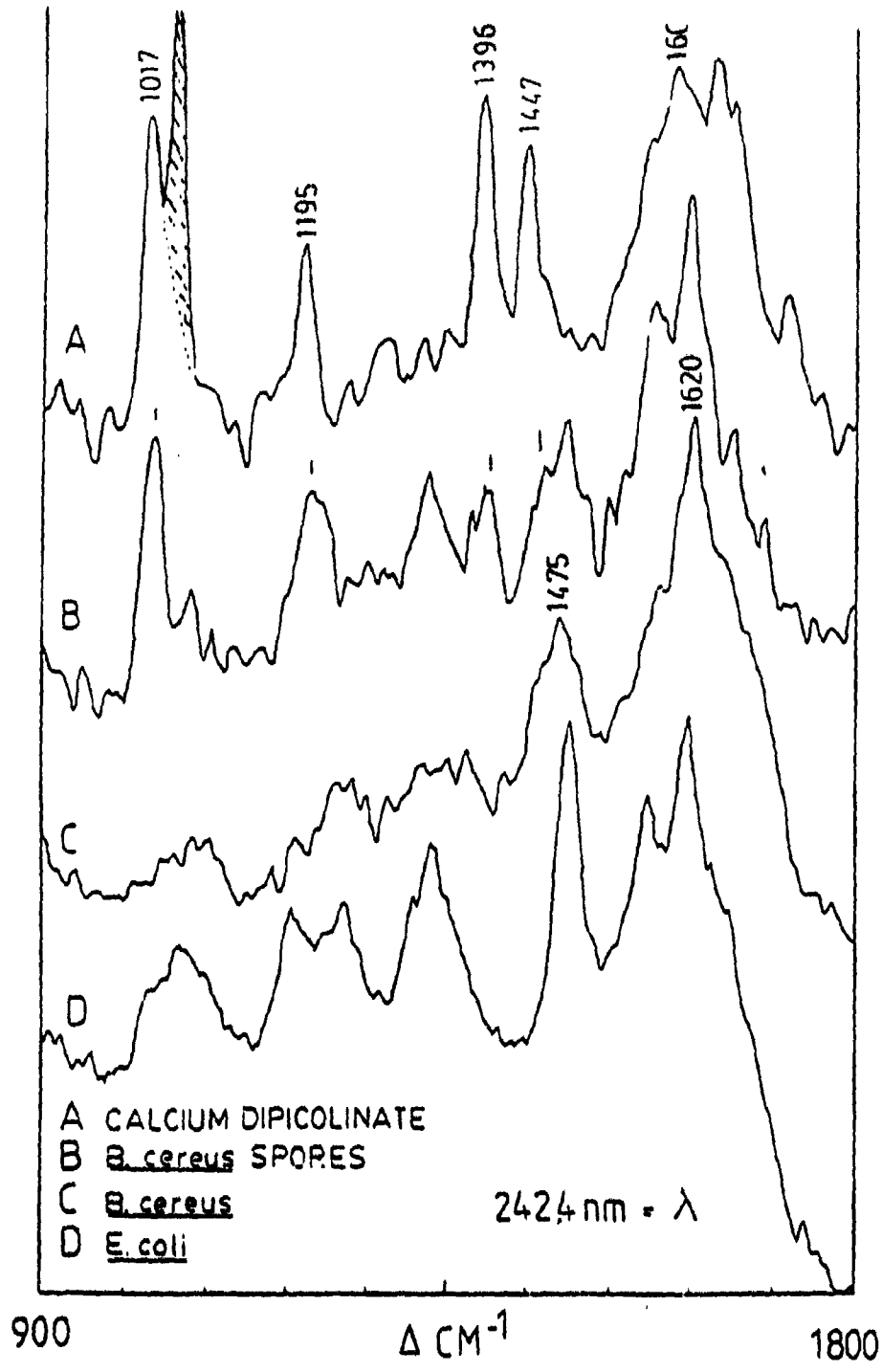

Fig. 6. A comparison of the 242 nm excited spectra of B. cereus spores with those of vegetative cells and calcium dipicolinate.

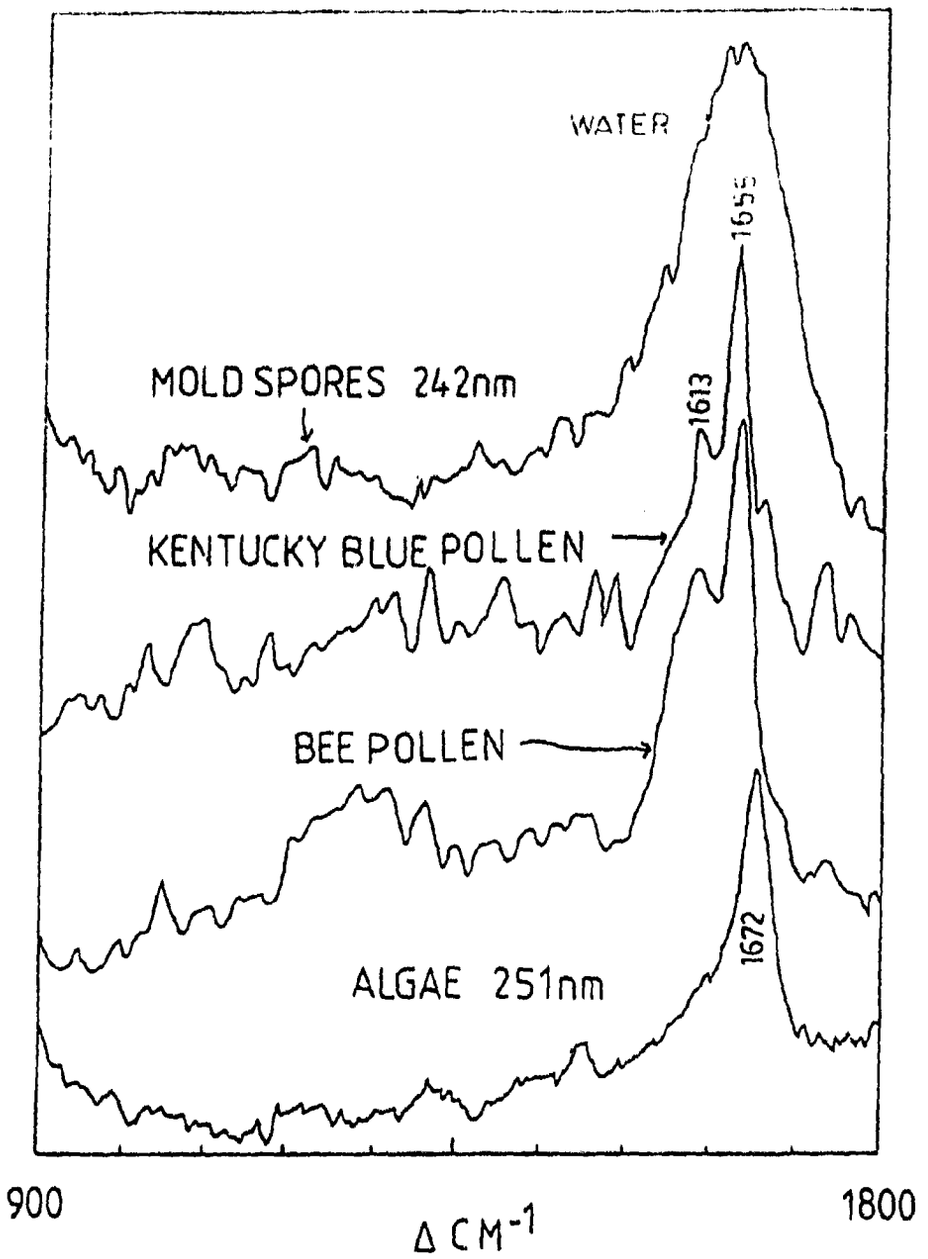

Fig. 7. A comparison of the resonance Raman spectra of representative pollens, mold spores and algae.

C. List of All Publications and Reports July 1984-present

1. "Rapid Detection of Microorganisms Using a Resonance Raman Microprobe." R. A. Dalterio, W. H. Nelson, J. Sperry and F. J. Purcell. Proc. 1984 Scientific Conference on Chemical Defense Research. CRDEC Nov. 1984.

2. "The Rapid Identification of Bacteria Using Time-Resolved Fluorescence and Fluorescence Excitation Spectral Methods." S. K. Brahma, M. P. Baek, D. Gaskill, R. K. Forcé, W. H. Nelson and J. Sperry, Appl. Spectrosc. 39, 869 (1985).

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